TLR4-Mediated Survival of Macrophages Is MyD88 Dependent and Requires TNF-α Autocrine Signalling

Eleuterio Lombardo, Alberto Alvarez-Barrientos, Beatriz Maroto, Lisardo Boscá and Ulla G. Knaus

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Modulation of macrophage survival is a critical factor in the resolution of inflammatory responses. Exposure to LPS protects innate immune cells against apoptosis, although the precise pathways responsible for prolongation of macrophage survival remain to be fully established. The goal of this study was to characterize the mechanism of TLR4-mediated survival of murine bone marrow-derived macrophages upon M-CSF withdrawal in more detail. Using a combination of knockout mice and pharmacological inhibitors allowed us to show that TLR4 and TLR2 stimulation promotes long-term survival of macrophages in a MyD88-, PI3K-, ERK-, and NF-κB-dependent manner. LPS-induced long-term, but not short-term, survival requires autocrine signaling via TNF-α and is facilitated by a general cytoprotective program, similar to that mediated by M-CSF. TLR4-mediated macrophage survival is accompanied by a remarkable up-regulation of specific cell surface markers, suggesting that LPS stimulation leads to the differentiation of macrophages toward a mixed macrophage/dendritic cell-like phenotype. *The Journal of Immunology, 2007, 178: 3731–3739.*

**TLR4-Mediated Survival of Macrophages Is MyD88 Dependent and Requires TNF-α Autocrine Signalling**

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Cell death by apoptosis is a process essential for normal development and maintenance of cell homeostasis. Alterations of normal cell survival mechanisms, leading to increased cell death or cell survival, are crucial for the development of a number of disorders such as cancer, autoimmune diseases, infectious diseases, and neurodegenerative diseases (1, 2).

Macrophages play an essential role in the innate immune response and normal tissue development by producing proinflammatory mediators and through clearance of pathogens or apoptotic cells by phagocytosis. Activation of macrophages by stimulation with cytokines or during the course of a microbial infection involves the modulation of a number of disorders such as cancer, autoimmune diseases, infectious diseases, and neurodegenerative diseases (1, 2).

Macrophages require M-CSF to maintain their viability and proliferation in vivo (15). In culture, bone marrow-derived macrophages (BMDM) and human monocyte-derived macrophages in vitro (16, 17). When M-CSF binds to its receptor, it triggers activation of PI3K, which in turn leads to activation of ERK and NF-κB (18, 19). It has been shown that PI3K is only required for M-CSF-mediated survival have not yet been fully characterized.

LPS is recognized by a TLR4/MD2/CD14 complex, which triggers the activation of two independent signaling cascades. The initiation of these pathways is mediated by different adaptor proteins as follows: one branch signals via Toll/IL-1R domain-containing adaptor protein and MyD88, and the other one via TRIF-related adaptor molecule and Toll/IL-1R domain-containing adaptor-inducing IFN-β (TRIF). The MyD88-dependent cascade is transmitted by the subsequent formation of a complex of IL-1R-associated kinases, TNFR-associated factor 6, and IFN regulator factor 5, leading to NF-κB and AP-1 activation. Trif, in contrast, relays its signals via a TNFR-associated factor 3-TANK binding kinase 1-IFN regulatory factor 3 axis in concert with a delayed NF-κB activation to induce type 1 IFNs. LPS stimulation also triggers the activation of small GTPases; the PI3K/Akt pathway; and the ERK, JNK, and p38 MAPK pathways. TLR4 activation results in the release of proinflammatory cytokines (TNF-α, IFN-β, IL-1β, etc.) and the induction of antiapoptotic genes, including members of the Bcl-2 and inhibitor of apoptosis family of proteins (14).

M-CSF (also known as CSF-1) is a myeloid growth factor controlling the survival, proliferation, and differentiation of monocytes and macrophages in vivo (15). In culture, bone marrow-derived macrophages (BMDM) and human monocyte-derived macrophages require M-CSF to maintain their viability and proliferation (16, 17). When M-CSF binds to its receptor, it triggers activation of PI3K, which in turn leads to activation of ERK and NF-κB (18, 19).

**Abbreviations used in this paper:** TRIF, Toll/IL-1R domain-containing adaptor-inducing IFN-β; BMDM, bone marrow-derived macrophage; cIAP, cellular inhibitor of apoptosis protein; TNOS, inducible nitric oxide synthase; KO, knockout; L929 CM, L929 cell-conditioned medium; LTA, lipoteichoic acid; MDDC, macrophage-derived dendritic cell; MnSOD, manganese superoxide dismutase; ox-LDL, oxidized low-density lipoprotein; PARP, poly(ADP-ribose) polymerase; PCNA, proliferating cell nuclear antigen; PI, phosphatidylinositol; SN, supernatant; WT, wild type; XIAP, X-linked inhibitor of apoptosis protein.
Macrophage survival, but not for macrophage proliferation (20, 21). M-CSF deprivation of macrophages induces cell cycle arrest and initiates rapid apoptosis (22). This process does not require caspase 8 activation, but proceeds via activation of the caspase 9/caspase 3 cascade and cytochrome c release from mitochondria (23). The observed resistance of macrophages to apoptosis when stimulated with LPS or oxidized low-density lipoprotein (ox-LDL) may depend on the expression of cellular inhibitors of apoptosis protein (cIAP2), Bcl-xL, and X-linked inhibitor of apoptosis protein (XIAP) (4, 23, 24).

Because the balance between apoptosis and survival is critical for macrophage function, we set out to characterize the mechanism of TLR4-mediated survival of BMDM upon M-CSF withdrawal. Our results show that TLR stimuli are equivalent to M-CSF and TNF-α in providing long-term protection against apoptosis. Multiple pathways, emanating from the MyD88 adapter, are essential for the antiapoptotic effect. One of the requirements for long-term survival is an autocrine loop involving TNF-α. During this survival process, macrophages acquire up-regulated cell surface markers, indicating a phenotypic change.

Materials and Methods

Chemicals
LPS (Escherichia coli, 0111:B4, ultrapure) and lipopolysaccharide (LTA) (Staphylococcus aureus) were obtained from List Biological Laboratories and InvivoGen, respectively. Murine TNF-α, IL-1β, IFN-γ, GM-CSF, M-CSF, and IL-4 were obtained from PeproTech. SN-50, SB203580, LY294002, U0126, JNK inhibitor II, and staurosporine were from EMD Biosciences. Polymyxin B sulfate, (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) MTT, propidium iodide (PI), sulfanilic acid, naphthoylethine, and xanthene were purchased from Sigma-Aldrich. Anti-Fas Jo-2 Ab was obtained from BD Pharmingen.

Mice
Wild-type (WT) C57BL/6 mice were purchased from The Jackson Laboratory. Mice on a C57BL/6 background with deficiency in MyD88 (MyD88−/−), TRIF (TRIF−/−), TNF-α (TNF−/−), and p55 TNF-α (p55−/−) were provided by K. Hoebel (The Scripps Research Institute, La Jolla, CA). Mice were housed in a specific pathogen-free environment in which all of the experiments were performed in accordance with Institutional Animal Care and Use Guidelines.

Cell culture
Bone marrow cells were isolated from femurs of 8- to 12-wk-old mice, as described elsewhere (25). Cells were seeded in bacterial dishes for 4 h in RPMI 1640 containing 10% FBS. Nonadherent cells were removed and cultured in the above medium containing 15% L929 cell-conditioned medium (L929 CM) as a source of M-CSF. After 7 days, cells were harvested using 10 mM EDTA (pH 7.2), seeded, and kept in RPMI 1640 containing 5% FBS for 16 h before the experiments.

Flow cytometry
A total of 3 × 106 BMDM from WT mice in RPMI 1640/5% FBS was treated with GM-CSF (20 ng/ml) and IL-4 (20 ng/ml), M-CSF (30% of L929 CM), or LPS (100 ng/ml) and maintained in culture for 7 days. To maintain differentiation to a dendritic cell phenotype, every 24 h additional GM-CSF and IL-4 at a concentration of 10 ng/ml were added. Cells were harvested and stained with the mAbs anti-MHC-II FITC, anti-CD80 PE, anti-F4/80 PE-Cy5, anti-CD11c PE-Cy7, and CD11b Cy5 (eBioscience). The appropriate mAb isotypic controls were included, with acquisition and analysis gates set accordingly. Data were acquired on a CyAn MLE-R cytometer (DakoCytomation) and analyzed with Summit cytometry version 3.1 software. Ten thousand events were counted for each analysis.

Metabolic activity
Macrophages were seeded at 105 cells/well in 24-well plates in 1 ml of RPMI 1640/5% FBS overnight. Cells were incubated at the indicated experimental conditions. At different time points, medium was removed, and 0.5 ml of MTT solution (0.5 mg/ml in RPMI 1640/5% FBS) was added to each well. After 30 min at 37°C, cells were washed once with PBS and 100 μl of DMSO was added. Spectrophotometric measurement at 570 nm was performed using a 96-well plate reader (iEMS Reader; Thermoelectron).

PI staining
Macrophages were seeded at 105 cells/well in 24-well plates in 1 ml of RPMI 1640/5% FBS overnight. After treatment, cells were harvested with trypsin-EDTA (0.25%) and stained with PI (0.005% in PBS for 15 min at room temperature). Cell death was analyzed by flow cytometry in a FACScan cytometer (BD Biosciences). Ten thousand events were counted for each analysis.

Cell viability
Quantification of the percentage of viable cells was performed using a dot plot of the forward scatter against the PI fluorescence by flow cytometry. Cells that were negative for PI and had normal size, morphology, and complexity were considered viable.

Immunoblotting
Cells were collected in radioimmunoprecipitation assay lysis buffer containing protease inhibitors, and protein concentrations were determined by bicinchoninic acid protein assay (Pierce). Lysates were separated by SDS-PAGE, transferred onto nitrocellulose membrane, and blotted with primary and secondary Abs, and proteins were visualized by ECL (GE Healthcare). Anticleaved poly(ADP-ribose) polymerase (PARP) (7C9), anti-Bcl-xL, anti-Bcl-2, and anti-Akt Abs were obtained from Cell Signaling Technology. Anti-manganese superoxide dismutase (MnSOD) (SOD-110) was obtained from StressGen Biotechnologies. Anti-XIAP was from BD Biosciences. Anti-cIAP2 (H-85) and anti-proliferating cell nuclear Ag (PCNA) were from Santa Cruz Biotechnology. Anti-β-actin was from Sigma-Aldrich.

Collection of treated medium
Macrophages were seeded at 5 × 106 cells/well in 10-cm dishes in 10 ml of RPMI 1640/5% FBS overnight. The next day, cells were left unstimulated or stimulated with LPS (100 ng/ml), and the medium was collected and frozen at different time points (supernatant [SN]). For the second stimulation, macrophages were seeded at 105 cells/well in 24-well plates in 1 ml of RPMI 1640/5% FBS overnight. The next day, medium of the previous experiment (SN) was treated with polymyxin B (10 μg/ml) for 90 min at 37°C to inhibit LPS before stimulation. Then the macrophage medium was replaced with treated medium (SN) for the second stimulation.

TNF-α complementation of macrophages
WT and TNF−/− macrophages were seeded in 24-well plates, as indicated above. The next day, cells were stimulated with LPS (100 ng/ml), and TNF-α was added at either 24 or 48 h poststimulation.

Results

LPS inhibits M-CSF withdrawal-mediated macrophage apoptosis
BMDM require M-CSF for survival, proliferation, and differentiation (15). To determine the effect of LPS on BMDM viability, macrophages that had been grown for 7 days in L929 CM as a source for M-CSF were kept overnight in fresh FBS-containing medium without M-CSF. The next day, treatment with M-CSF (or 10% L929 CM), LPS, or IFN-γ, a previously identified prosurvival factor (22), was started and cell death was determined 24 h poststimulation. Preliminary results indicated that L929 CM was similarly protective against apoptosis as purified M-CSF (data not shown). Thus, 10% L929 CM was used instead of M-CSF throughout all of the experiments. BMDM underwent apoptosis in the absence of M-CSF, as determined by PI staining and detection of cleaved PARP, and showed decreased cell viability and metabolic activity (Fig. 1, A–D). However, treatment of cells with LPS inhibited these apoptotic features to a similar degree as observed with M-CSF and IFN-γ. LPS-induced survival was not accompanied by proliferation because the proliferation marker PCNA showed no significant changes in BMDM stimulated for 24 or 48 h with LPS (Fig. 1E).

LPS promotes long-term survival of macrophages
To investigate whether the antiapoptotic effect of LPS on macrophages could be sustained over a longer period of time, the viability of LPS-stimulated BMDM was monitored over several days.
LPS led to long-term survival of at least 5 days after the initial M-CSF withdrawal with <25% of cells gated as PI positive. Unstimulated macrophages showed increasing numbers of PI-positive cells (~85% by day 5), indicating massive apoptosis (Fig. 2A). Similar results were obtained in cell viability and metabolic activity assays (data not shown). LPS, in contrast, was effective as an antiapoptotic stimulus over a wide range of concentrations (10–1000 ng/ml).

Because LPS was continuously present during these experiments, we wanted to determine whether the duration of LPS exposure is essential for macrophage survival. BMDM were stimulated with LPS, and medium was removed at different time points (6 and 24 h) or was kept for the full 5 days. For the first two time points, the medium was replaced after the indicated time and cells were incubated in fresh medium until day 5. When BMDM viability was determined by PI staining at these time points, complete medium removal after 6 or 24 h led to short-term protection (for 48 h). Efficient long-term protection was only provided when the medium was retained over the complete time course of 5 days (Fig. 2B). The fact that the original medium needs to be kept in culture to provide long-term survival suggests that LPS-mediated TLR4 signaling triggers the expression of antiapoptotic proteins and potentially the release of antiapoptotic mediators into the medium. Continuous LPS treatment of cells usually leads to down-regulation of TLR4 and tolerance, and should therefore not afford long-term protection from apoptosis (26).

To further study survival induction through TLR4, we investigated whether LPS stimulation blocks apoptosis induced by other...
agents. Thus, BMDM were stimulated with LPS in the presence of FBS and M-CSF (5% L929 CM) for 24 h before an additional 24-h treatment with dexamethasone (10 μg/ml), anti-Fas Ab (20 μg/ml), staurosporine (1 μM), or doxorubicin (10 μM). TLR4 activation blocked cell death induced by dexamethasone and Fas ligation, but had no effect on the cytotoxic effects of doxorubicin or staurosporine (Fig. 2C).

Antiapoptotic TLR4 signaling is MyD88 dependent and requires PI3K, ERK, and NF-κB pathways

Activation of TLR4 leads to stimulation of MyD88-dependent and MyD88-independent pathways. To identify which one of these pathways is required for LPS-mediated survival in the absence of M-CSF, BMDM from MyD88 and TRIF-deficient mice were stimulated with LPS, and cell death was determined at different time points by PI staining. Whereas macrophages derived from WT or TRIF−/− mice showed long-term survival in the presence of LPS, MyD88-deficient macrophages were only partially protected in the first 24 h and became apoptotic thereafter (Fig. 3A). These results indicate that MyD88-dependent signaling is required for macrophage survival after M-CSF withdrawal.

TLR4/MyD88-mediated signaling activates multiple protein kinases and transcription factors. To evaluate which of these pathways mediates the antiapoptotic effect, we used pharmacological inhibitors to block p38 kinase, JNK, ERK, PI3K, and NF-κB activity and assessed their effect on LPS-induced survival using an assay that measures the metabolic activity of cells (Fig. 3B). Inhibition of p38 kinase or JNK did not significantly interfere with LPS-mediated survival, whereas inhibition of ERK, PI3K, or NF-κB partially reduced macrophage survival. The combined inhibition of PI3K and NF-κB, or PI3K and ERK, synergistically reduced LPS-mediated survival. Neither the PI3K nor the ERK inhibitor had any inhibitory effect on LPS-induced IκBα degradation (Fig. 3C). These data indicate that multiple pathways contribute to macrophage survival downstream of MyD88.

Role of pathogen-associated molecular patterns and cytokines in M-CSF deprivation-induced apoptosis

Our data to date indicate that LPS is able to induce macrophage long-term survival. To compare this LPS effect with other TLR ligands or cytokines, M-CSF was again removed from macrophage medium before various stimuli, including M-CSF, LPS, LTA (TLR2 ligand), IFN-γ, IL-1β, or TNF-α, were added. Cell death was determined after 1, 2, 3, and 5 days by PI staining, cell viability, and metabolic activity assays (Fig. 4, A–C). As expected, in the absence of M-CSF, macrophages succumbed to apoptosis rapidly. However, the addition of LTA or TNF-α induced long-term macrophage survival reminiscent of LPS. IFN-γ, which was previously reported to inhibit apoptosis after M-CSF withdrawal (25), induced short-term survival for 48 h, but failed to protect thereafter. The proinflammatory cytokine IL-1β did not rescue macrophages and promoted cell death to a similar extent as observed in unstimulated macrophages. In conclusion, TLR2 and TLR4 ligands, as well as TNF-α, play a sustained protective role for M-CSF-starved macrophages.

Expression levels of antiapoptotic proteins of the Bcl-2 and inhibitor of apoptosis protein families (i.e., XIAP, cIAP2, Bcl-xL) are essential in maintaining macrophage survival in vitro and in vivo (4, 23, 24). Activation of PI3K and NF-κB signaling cascades rapidly induces the up-regulation of antiapoptotic genes, including Bcl-2, Bcl-xL, cIAP2, and XIAP. To gain insight into the mechanisms involved in short-term survival of BMDM, we compared the expression levels of Bcl-2, Bcl-xL, XIAP, and cIAP2. We also included in our analysis the mitochondrial antioxidant enzyme MnSOD, as well as the activation of the prosurvival kinase Akt (Fig. 4D). Control macrophages deprived of M-CSF displayed reduced levels of XIAP, cIAP2, and Bcl-2, and reduced Akt phosphorylation when compared with macrophages maintained with M-CSF. This suggests that apoptosis might be due to the decreased levels of those antiapoptotic proteins. MnSOD protein expression, although highly induced by LPS and TNF-α, was very similar between unstimulated and M-CSF-starved macrophages. XIAP and cIAP2 were increased by LPS, TNF-α, and IFN-γ, but not by IL-1β, when compared with
unstimulated cells. The expression levels of Bcl-xL and Bcl-2 did not correlate with a pro- or antiapoptotic phenotype. To summarize, we did not observe a close correlation between the expression or activation of signaling molecules tested and the outcome, i.e., macrophage survival or apoptosis. This suggests that there may not be one specific protein responsible for TLR4-mediated survival, but that rather a generalized, multifactorial program is induced to maintain metabolic activity and protection from caspase activation. Due to the limitations of cell numbers in nonprotected conditions, we were unable to analyze expression levels or kinase activation at later time points.

Polymyxin B-treated LPS-conditioned medium supports macrophage survival

As shown in Fig. 2B, LPS-induced long-term survival could be mediated by an autocrine loop, perpetuated by survival factors released into the medium after TLR4 stimulation. Our data also indicate that TNF-α has a similar antiapoptotic role for macrophages as LPS. If this hypothesis is valid, conditioned medium from LPS-stimulated macrophages should have the capability to support macrophage survival even after polymyxin treatment. Polymyxin B, a polycationic cyclic peptide, binds to the lipid moiety of LPS and inactivates its biological activity (13). To test this, conditioned medium obtained from M-CSF-deprived BMDM without undergoing stimulation or with LPS stimulation was harvested, treated with 10 μg/ml polymyxin B sulfate, and added to a different set of macrophages that had been deprived of M-CSF for 24 h. As a control for polymyxin B efficiency, LPS was either left untreated (UN) or treated with LPS (100 ng/ml). Twenty-four hours later, vehicle or polymyxin B (10 μg/ml) was added and kept in the medium. Cell death was determined at day 5 by PI staining. One representative experiment of three is shown.
stimulation did not inhibit LPS-mediated survival (Fig. 5B). This demonstrates that survival is not due to constant LPS stimulation and that TLR4 activation leads to the release of survival factors into the medium that seem to be responsible for LPS-induced long-term survival.

**TLR4-dependent survival is not mediated by NO or M-CSF release**

We next sought to identify soluble factors mediating the survival effect of LPS. Upon TLR4 activation, macrophages up-regulate inducible nitric oxide (iNOS) and release NO. Low concentrations of NO have been shown to attenuate apoptosis in macrophages, in part through the activation of NF-κB and AP-1 (27, 28).

To determine whether NO plays a role in LPS-mediated protection during M-CSF withdrawal, BMDM were stimulated with LPS in the presence or absence of the specific iNOS inhibitor 1,400 W and cell death was determined by PI staining at different time points. Although NO production was completely blocked by the presence of this inhibitor, LPS-mediated survival was not affected at any time point (data not shown).

**TLR4 activation may lead to the induction of M-CSF** (29). To explore whether M-CSF induction by LPS plays a role in LPS-mediated macrophage survival, we used a M-CSF receptor-blocking Ab, anti-CD115 (30). BMDM were stimulated with LPS in the presence of IgG isotype control or anti-CD115, and macrophage survival was followed for 5 days. Every 24 h, another aliquot of the Ab was added to the medium to maintain blockage of M-CSF signaling. LPS-mediated long-term survival in both anti-CD115- and IgG isotype-treated macrophages mimicked that obtained with LPS alone (data not shown). These results indicate that the putative release of M-CSF does not cause LPS-induced survival.

**TLR4-mediated long-term survival of macrophages requires TNF-α**

TNF-α is highly produced by macrophages upon TLR4 activation and supports long-term macrophage survival (Fig. 4, A–C). These data suggested that an autocrine loop involving TNF-α may play a central role during TLR4-mediated survival of macrophages. Therefore, BMDM from TNF-α-deficient and p55 TNFR-deficient mice were prepared and stimulated with LPS. Cell death was determined by PI staining at days 2 and 5 after stimulation and compared with WT BMDM that were subjected to the same treatment. No significant difference in survival was observed at day 2 between WT, TNF−/−, and p55−/− macrophages (Fig. 6A), whereas LPS-stimulated TNF−/− as well as p55−/− macrophages displayed at day 5 increased apoptosis (52 and 49%, respectively) when compared with WT macrophages (27%).

To further confirm the contribution of TNF-α to TLR4-mediated survival, medium from WT or TNF−/− macrophages, which had been stimulated for 24 h with LPS, was harvested and treated with polymyxin B, as indicated above. The potential of these different media to delay apoptosis was compared by adding them to untreated WT, TNF−/−, and p55−/− BMDM. Analysis of cell death by PI staining was performed on days 2 and 5 after medium addition (Fig. 6B). Consistent with data shown in Fig. 6A, WT- and TNF−/−-derived medium (SN WT and SN TNF−/−, respectively) were able to support macrophage survival at day 2 in any of the macrophage populations tested. However, at day 5, the protective effect of WT medium was diminished in TNF−/− (34% of PI-positive cells) and p55−/− macrophages (24%) when compared with WT macrophages (15%), indicating that TNF-α signaling is required. Furthermore, in line with these observations, medium derived from TNF−/− BMDM was less potent in supporting survival of WT-, TNF−/−-, and p55−/−-derived macrophages at day 5 (Fig. 6B).

We then asked whether addition of exogenous TNF-α to LPS-stimulated TNF−/− BMDM would reconstitute long-term survival to the WT levels. When TNF-α was added after 24 or 48 h to the medium of LPS-stimulated WT or TNF−/− macrophages, the protection from apoptosis was restored in TNF−/− BMDM. These results indicate that LPS-induced short-term survival is independent of TNF-α, whereas long-term survival is at least partially dependent on autocrine signaling by TNF-α. TNF-α by itself promotes survival at least partially via NF-κB-dependent gene transcription because the cell-permeable peptide inhibitor SN50 caused a 50% decrease in day 2 and day 5 survival (data not shown).

**TLR4-mediated long-term survival of macrophages is accompanied by a phenotypic change**

Activation of immune cells often triggers a differentiation program, which can extend the life span of cells. In view of the prolonged survival of macrophages upon TLR4 stimulation, we hypothesized that LPS could induce the differentiation of BMDM.
toward a more dendritic-like phenotype. To test this hypothesis, BMDM were cultured for 7 additional days in the presence of GM-CSF (30% L929 CM), LPS (100 ng/ml), or GM-CSF plus IL-4 (10 ng/ml each). On day 7, cells were stained with Abs for the indicated surface markers and analyzed by flow cytometry. Numbers within the histograms represent the mean change of fluorescence intensity when compared with unstimulated control at day 6. Data shown are representative of three different experiments.

Discussion

Inflammation is the response of the organism to tissue remodeling or infection by pathogens. The inflammatory response, in which macrophages are key players, is subject to a fine-tuned regulation to avoid prolonged and chronic inflammation. Apoptosis is a key regulator of homeostasis, and thus, imbalance of cell growth and cell death is associated with the development of a number of diseases (1, 2). Certain bacterial- or cytokine-derived ligands impart macrophages with an increased resistance against apoptotic triggers. This resistance to apoptosis is essential for macrophages to function within a proinflammatory environment. In this regard, relatively little is known about the mechanisms involved in LPS-mediated survival of BMDM.

Our study focused on the mechanisms triggered by LPS, which lead to primary BMDM survival upon M-CSF deprivation. We found that even very low concentrations of LPS induced prolonged macrophage survival substantially, indicating that LPS is a potent antiapoptotic stimulus for macrophages. To determine whether LPS-mediated survival is accompanied by proliferation, we analyzed the expression of the mitosis marker PCNA. PCNA was highly induced in M-CSF-treated cells, but not induced by LPS, indicating that proliferation does not play any role in the prosurvival effect of LPS.

Using specific pharmacological inhibitors and macrophages deficient in the TLR adapter proteins MyD88 or TRIF, we demonstrated that MyD88 is required for macrophage survival. Moreover, the combined inhibition of PI3K-NF-κB and PI3K-ERK signaling impacted LPS-mediated survival. These observations are in agreement with those reported by others using LPS-stimulated cardiomyocytes, neutrophils, and monocytes (10–12), as well as ox-LDL-stimulated macrophages (23). This suggests that PI3K, ERK, and NF-κB are pivotal mediators of LPS-induced survival in several cell types. Stimulation with M-CSF leads to the activation of these three signaling cascades (15), suggesting that LPS may induce macrophage survival by partially mimicking M-CSF action.

The prosurvival effect of LPS was compared with other TLR ligands (LTA) and proinflammatory cytokines, such as TNF-α, IFN-γ, and IL-1β. The tested stimuli fell into the three following categories: 1) those able to induce a long-term survival for at least 5 days (LPS, LTA, and TNF-α); 2) those able to induce short-term survival for 48 h (IFN-γ); and 3) those that failed to induce macrophage survival (IL-1β).

The differentiation of macrophages from bone marrow progenitors, which is orchestrated by M-CSF, is associated with a steady and gradual increase in the levels of XIAP, cIAPs, Bcl-2, and Bcl-xL (24). A role for XIAP, cIAP-2, Bcl-2, and Bcl-xL in inhibiting macrophage apoptosis is well documented. Increased Bcl-2 and Bcl-xL expression levels correlate with macrophage survival induced by M-CSF, GM-CSF, apoptotic cells, and ox-LDL (23, 32–34). LPS induces XIAP and cIAP-2 expression, which is essential for LPS- or NO-mediated macrophage survival (4, 35, 36). In addition, PI3K-dependent Akt activation and subsequent inhibition of Bad play a major role in immune cell survival (11, 37, 38). Treatment with LPS or TNF-α prevented the general decrease in antiapoptotic proteins after M-CSF withdrawal; however, our data failed to clearly determine a candidate responsible for prolongation of macrophage survival by LPS, and suggest that a combination of several prosurvival factors is required for long-term protection.

Three different experimental approaches using WT, TNF−/−, and p55−/− macrophages allowed us to demonstrate that LPS-mediated long-term, but not short-term, survival requires autocrine signaling by TNF-α, as follows: 1) comparison of LPS-induced survival in WT and knockout (KO) macrophages; 2) comparison of the capacity of LPS-conditioned medium derived from WT and TNF−/− macrophages in promoting survival; and 3) reconstitution of LPS-mediated long-term survival in TNF−/− macrophages by addition of exogenous TNF-α. However, factors other than TNF-α must be required because partial macrophage survival is still achieved in both TNF−/− and p55−/− macrophages. We could not confirm a role for LPS-produced M-CSF or TRIF-mediated NO generation as antiapoptotic mediators. It is conceivable that soluble factors such as IL-6 could promote the early survival effect and potentially sensitize the macrophage for TNF-α as the long-term survival factor (39). A biphasic response would suggest a highly
regulated and temporal role for different cytokines in immune cell survival. TNF-α is believed to mediate many of the pathologic effects of LPS in inflammation and septic shock, and recent data showed that prolonged macrophage survival plays a critical role in LPS-induced endotoxin shock (4).

LPS can promote, similar to TNF-α, survival and apoptotic pathways, and consequently, conflicting results exist in regard to pro- and anti-apoptotic effects of TL4 stimulation. Our data as well as reports by others support LPS-induced survival signaling in several cell types (10–12, 40). In fact, apoptosis studies using M-CSF-deprived BMDM often use LPS together with MAPK inhibitors for efficient down-regulation of transcriptionally activated survival pathways (41). In contrast to our findings are reports by Celada and colleagues (42, 43), who demonstrated LPS-mediated early apoptosis (up to 6 h), followed by autocrine, TNF-α, and p55 TNFR KO mice. We also thank Susana Rodríguez for excellent technical assistance and Katrina Schreiber for administrative and graphical assistance.

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Disclosures
The authors have no financial conflict of interest.

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21. Murray, J. T., G. Craggs, L. Wilson, and S. Kellie. 2000. Mechanism of phos- phatidylinositol 3-kinase-dependent increases in BAC1.2F5 macrophage-like cell density undergo apoptosis upon M-CSF deprivation faster than when cultured at a high density), and the culture conditions during the initial growth and differentiation of macrophages from bone marrow, account for the above mentioned differences. As TL4 activation can trigger both antiapoptotic and proapoptotic cascades, it is reasonable to suppose that certain conditions favor one over the other pathway. It is interesting to note that Boggs et al. (48) correlated the redox status of mouse macrophages to survival vs apoptosis.

LPS-activated macrophages alter their phenotype in culture, possibly to maintain high metabolic activity. In our conditions, these macrophages acquired higher expression of macrophage markers and certain cell surface molecules that are usually the hallmark of immature dendritic cells. Further work is necessary to assess how these phenotypic changes alter the capacity of these macrophages in respect to their microbicidal activity or their ability to influence the adaptive immune system.

LPS in MACROPHAGE SURVIVAL

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